Immunomodulatory Mediators from Pollen Enhance the Migratory Capacity of Dendritic Cells and License Them for Th2 Attraction[1]

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The immune response of atopic individuals against allergens is characterized by increased levels of Th2 cytokines and chemokines. However, the way in which the cytokine/chemokine profile is matched to the type of invading allergen, and why these profiles sometimes derail and lead to disease, is not well understood. We recently demonstrated that pollen modulates dendritic cell (DC) function in a way that results in an enhanced capacity to initiate Th2 responses in vitro. Here, we examined the effects of aqueous birch pollen extracts (*Bet.*-APE) on chemokine receptor expression and chemokine production by human monocyte-derived DCs. *Bet.*-APE strongly induced expression and function of CXCR4 and reduced CCR1 and CCR5 expression on immature DCs. In addition, DC treatment with *Bet.*-APE significantly reduced LPS-induced production of CXCL10/IP-10, CCL5/RANTES; induced CCL22/macrophage-derived chemokine; and did not significantly change release of CCL17/thymus and activation-regulated chemokine. At a functional level, *Bet.*-APE increased the capacity of LPS-stimulated DCs to attract Th2 cells, whereas the capacity to recruit Th1 cells was reduced. *Bet.*-APE significantly and dose-dependently enhanced intracellular cAMP, suggesting that water-soluble factors from pollen grains bind a $G_{\alpha s}$-protein-coupled receptor. E_1-Phytoprostanes were identified to be one player in the Th2-polarizing potential of aqueous pollen extracts. In summary, our results demonstrate that pollen itself releases regulatory mediators which generate a Th2-promoting micromilieu with preferential recruitment of Th2 cells to the site of pollen exposure.

Dendritic cells (DCs)[3] are professional APCs capable of Ag transport and presentation in secondary lymphoid organs, which is crucial for the initiation and maintenance of T cell-mediated immune responses (1, 2). They reside in the periphery in an immature state, taking up pathogens or allergens through pinocytosis or receptor-mediated endocytosis, leading to the induction of DC maturation. DCs undergo a maturation process, induced by inflammatory cytokines, bacterial or viral products, which leads to their migration to lymph nodes, where they efficiently attract and activate T cells (3).

The trafficking of immature DCs to sites of inflammation and of mature DCs to the T cell area of secondary lymphoid organs is regulated by the expression of different chemokines and chemokine receptors (4, 5). Immature DCs express inflammatory chemokines (CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES, and CCL20/MIP-3α) and chemokine receptors that bind to inflammatory chemokines (CCR1, CCR2, CCR5, CCR6, and CXCR1). On maturation, DCs down-regulate the inflammatory chemokines and their receptors and up-regulate chemokines such as CXCL10/IP-10, CCL17/thymus and activation-regulated chemokine (TARC), CCL18/pulmonary and activation-regulated chemokine, CCL19/MIP-3β, CCL22/ macrophage-derived chemokine (MDC), and the chemokine receptor CCR7, ligand of the lymph node-derived chemokines CCL19/MIP-3β and CCL21/secondary lymphoid tissue chemokine (6). PGE_2 has been shown to up-regulate CCR7 and CXCR4 on mature monocyte-derived DCs (MoDC) and license their migration to CCL19/MIP-3β and CCL21/secondary lymphoid tissue chemokine and CXCL12/SDF-1α (7).

In allergic individuals, the uptake of allergens by dendritic cells ends in an allergen-specific Th2-biased immune response that ultimately leads to clinical manifestations of IgE-mediated hypersensitivity (8). Allergen-specific Th2 cells are the key orchestrators of allergic reactions, initiating and propagating inflammation through the release of a number of Th2 cytokines such as IL-4 and IL-13. Although the biology of Th2 cells in allergy is well understood, little is known about the mechanisms that control the initial Th2 polarization in response to exogenous allergens.

In the context of allergy, pollen grains have simply been regarded as allergen carriers, and little attention has been devoted to

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[3] Abbreviations used in this paper: DC, dendritic cell; TARC, thymus and activation-regulated chemokine; MDC, macrophage-derived chemokine; MoDC, monocyte-derived DC; PPE_1, pollen-associated phytoprostane; *Bet.*-APE, *Betula alba* L. aqueous pollen extract.

further compounds of pollen. However, individuals are rarely exposed to purified allergens, but rather particles releasing the allergen such as pollen grains or pollen-derived granules (9–11).

We recently demonstrated that pollen, under physiologial exposure conditions, release not only allergens but also bioactive lipids that activate human neutrophils and eosinophils in vitro (12–14). Furthermore, pollen-associated phytoprostane (PPE_1) reduces in human MoDCs the LPS/CD40L-induced production of IL-12 p70 leading to a Th2 induction in naive heterogeneic T-lymphocytes (15). Here we describe the ability of *Betula alba* L. aqueous birch pollen extracts (*Bet.*-APE) to affect chemokine receptor expression and chemokine release of human DCs that can affect the DC capacity to home to lymphoid organs and to attract Th2 cells. In functional migration assays, we found that stimulation of DCs with *Bet.*-APE enhanced migration of type 2 T cells, suggesting an increased capacity to amplify type 2 immune responses. Furthermore, we provide data pointing to a cAMP-related mechanism leading to the observed effects. Understanding the mechanisms that regulate DC function after contact with allergen carriers will ultimately benefit the development of new therapeutic strategies in preventing and treating allergy.

Materials and Methods

Reagents and Abs

Human rIL-4 was obtained from Promocell, human rGM-CSF was from Essex, and soluble CD40L was from Alexis. Purified LPS (*Escherichia coli* K235-derived LPS; <0.008% protein) was kindly provided by Dr. Stephanie Vogel (Department of Microbiology and Immunology, University of Maryland, Baltimore, MD). Anti-CD4 and anti-CD45RA microbeads were from Miltenyi Biotech.

Preparation of Bet.*-APE*

Birch pollen grains (*B. alba* L.) were obtained from Allergon. *Bet.*-APEs were generated by incubation of pollen grains in RPMI 1640 (30 mg/ml) for 30 min at 37°C followed by centrifugation (20 min at 3345 × *g*) and sterile filtration (0.2 μm). In a previous study, this procedure was shown to be the most effective to release pollen-associated lipid mediators from pollen (12). LPS was measured by *Limulus* amebocyte lysate assay (Cambrex Bio Science). To deplete LPS, *Bet.*-APE were eluted over polymyxin B columns (Pierce), leading to LPS concentrations below the detection limit of the assay (<0.05 endotoxin units (EU)/ml). LPS-depleted *Bet.*-APE was used for all subsequent experiments.

MoDCs

Healthy, nonatopic blood donors were characterized by screening for total and specific IgE for common allergens as recently described (15). All volunteers were without medication for at least 15 days before blood sampling. The ethical committee of the Technical University of Munich approved the study and volunteers were enrolled in the study after written informed consent. MoDCs were prepared from peripheral blood of healthy individuals, as described recently (16). In brief, adherent PBMC (>90% pure $CD14^+$ cells) were cultured at 1×10^6 cells/ml in RPMI 1640 supplemented with 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 2 mM L-glutamine, 0.05 mM 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Invitrogen Life Technologies) supplemented with 10% FBS, 500 U/ml human rGM-CSF (Tebu Bio), and 500 U/ml human rIL-4 (Promocell; complete DC medium) at 37°C under 5% CO_2. At day 5, cells (>95% $CD1a^+$, $CD14^-$) were harvested and recultured in complete DC medium for 24 h at 37°C with or without indicated stimuli in the presence or absence of LPS (100 ng/ml) or soluble CD40L (1 μg/ml; Alexis) followed by addition of a cross-linker (1 μg/ml; Alexis). PGE_2 was used as control (1×10^{-6} M).

Generation of polarized Th1 and Th2 cell clones

Human $CD4^+CD45RA^+$ T cells were purified from nonadherent PBMC from healthy nonatopic donors using MACS column separators with anti-CD4 and anti-CD45RA microbeads (Miltenyi Biotec). Differently stimulated MoDC (24 h) were washed and cocultured with MACS-purified allogeneic naive $CD4^+CD45RA^+$ T cells (1×10^5 cells/well) in complete RPMI with autologous 5% human serum. LPS-activated DC (100 ng/ml, 24 h) were used to generate Th1 polarized T cell lines. Th2-polarized T

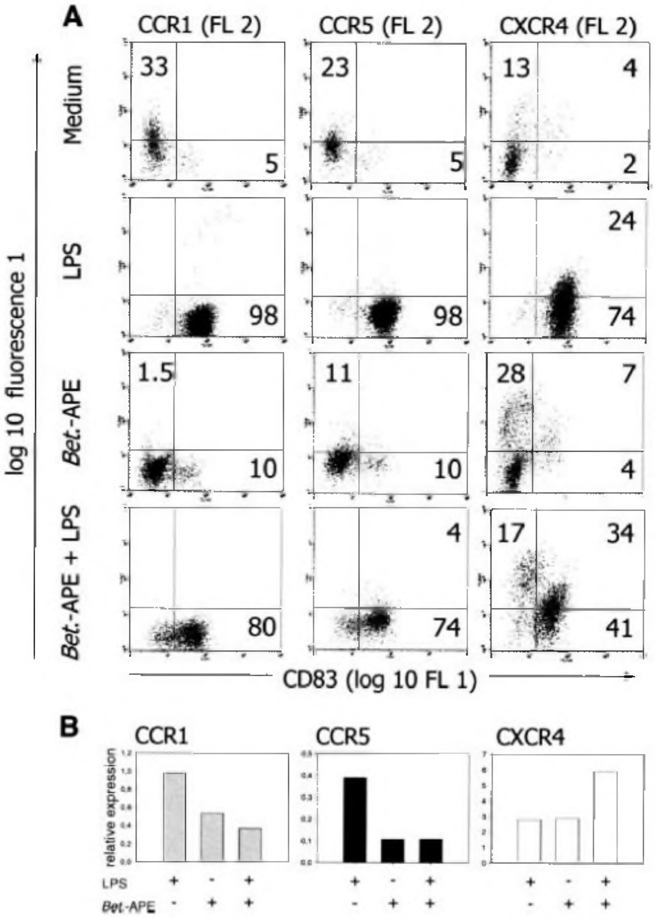

FIGURE 1. *Bet.*-APE reduces CCR1 and CCR5 expression while CXCR4 is induced on human MoDCs. *A*, MoDCs were stimulated on day 5 with LPS (100 ng/ml), *Bet.*-APE (3 mg/ml), or LPS and *Bet.*-APE or left untreated. After 24 h, DCs were stained with mAbs for CCR1, CCR5, and CXCR4 (FL2) and CD83 (FL1) as maturation marker and analyzed by flow cytometry. Shown is one representative experiment for three performed. *B*, Immature MoDCs were stimulated on day 5 for 6 h with LPS and *Bet.*-APE alone or in combination, as described in this legend, and then extracted for RNA. Real-time PCR was performed as described in *Materials and Methods*. Data are expressed as relative expression ($2^{(-\Delta\Delta CT)}$). Values are for one representative experiment of three performed.

cells were generated by using DCs that were activated (24 h) with LPS in the presence of PGE_2 (10^{-6} M; Alexis). In addition, neutralizing anti-IL-12 mAb (10 μg/ml; BD Biosciences) was added at the beginning of the T cell/DC coculture to generate a maximal Th2 polarization. The T cell lines were cloned after 10 days by limited dilution. As analyzed by flow cytometry, the Th1 clones used in the migration assay were strongly positive for CXCR3 and CCR5 and negative of CCR4 and CRTh2, whereas Th2 clones were negative for CCR5 and CXCR3 and positive for CCR4 and CRTh2 (Fig. 5; all Abs from BD Biosciences).

Flow cytometry of DCs and T cells

Surface expression of DC maturation marker CD83 and chemokine receptors of DCs were analyzed using multicolor flow cytometry. In brief, DCs, either untreated or stimulated for 24 h with LPS in the presence or absence of pollen extracts, were harvested, washed, and suspended in cold PBS containing 5% FCS and 0.02% NaN_3 and then incubated with saturating concentrations of FITC-conjugated mAb (CD83), and PE-conjugated mAb (CCR1, CCR5, and CXCR4, all from BD Biosciences). Matched isotype control mAb were used in control samples. Stained cells were analyzed using a FACSCalibur flow cytometer equipped with CellQuest software (BD Biosciences). Propidium iodide-permeable (nonviable) cells were excluded from analysis.

Quantitative mRNA analysis

Total RNA was extracted from purified DC after 6 h of incubation with the indicated stimuli using peqGOLD RNAPure buffer (Peqlab). RNA was

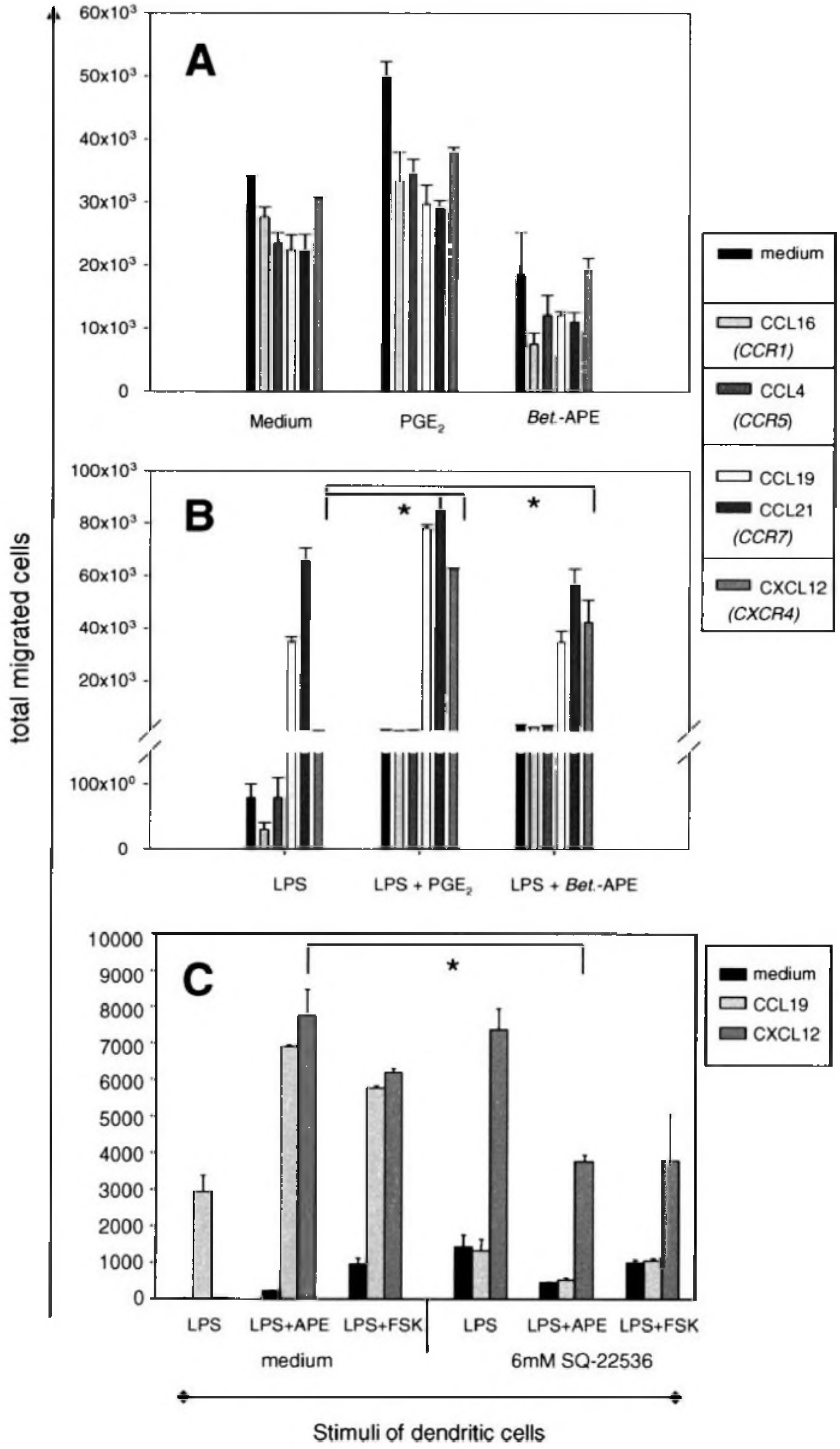

FIGURE 2. *Bet.*-APE augment the response of LPS-stimulated DCs to the CXCR4 ligand CXCL12 by a cAMP-dependent mechanism. MoDCs were incubated for 24 h with medium, *Bet.*-APE (3 mg/ml), PGE_2 (1×10^{-6}) (*A*), with LPS, LPS plus PGE_2, LPS plus *Bet.*-APE (*B*), or with LPS, LPS plus *Bet.*-APE and LPS plus forskolin (FSK) in the absence or presence of adenylyl cyclase inhibitor SQ-22536 (*C*). Stimulated DCs were processed for migration assays in 24-well Transwell chambers. As migratory stimuli the following chemokines were chosen: 1 μg/ml CCL16 (ligand for CCR1); 100 ng/ml CCL4 (ligand for CCR5); 30 ng/ml CCL21; 100 ng/ml CCL19 (both ligands for CCR7); and 10 ng/ml CXCL12 (ligand for CXCR4). Data are expressed as total migrated cells ± SD. Values are for one representative experiment of five performed in triplicate. *, Significant changes of DC migration ($p \leq 0.05$).

reverse transcribed using random hexamer primers (Roche). PCRs for the chemokine receptors CCR1, CCR5, CXCR4 and the chemokines CXCL10, CCL5, CCL22, and CCL17 (Assay on demand; Applied Biosystems) were run on an ABI PRISM 7700 Sequence Detection System device (Applied Biosystems Division of PerkinElmer) using the following program: 10 min at 94°C followed by 40 cycles of 15 s at 95°C, and 60 s at 55°C; 18s RNA served as housekeeping gene.

Migration assay

Chemotaxis of DCs and T cells was evaluated by measuring their migration through 5-μm-pore polycarbonate filters in 24-well Transwell chambers (Corning Costar), as described previously (13). Briefly, the chemotactic property of DC supernatants was evaluated by adding 10^5 T cells suspended in complete RPMI with 0.5% BSA to the top chamber and various dilutions of the supernatants (0.6 ml) to the bottom chamber of a Transwell insert (Costar). To analyze the role of cAMP-induction in the chemokine receptor regulation by *Bet.*-APE, day 5 MoDC were preincubated for 1 h in complete medium (+GM-CSF/IL-4) in the absence or presence of 6 mM adenylyl cyclase inhibitor SQ-22536 (Calbiochem). The cells were then stimulated with medium, 100 ng/ml *E. coli* LPS, *Bet.*-APE (10 mg/ml, 25% v/v), or 10 μM forskolin (Sigma-Aldrich). The stimuli *Bet.*-APE and forskolin were applied either alone or in combination with LPS. After 24 h, cells were harvested, washed twice, and resuspended in complete medium at a density of 1×10^6 cells/ml. For the

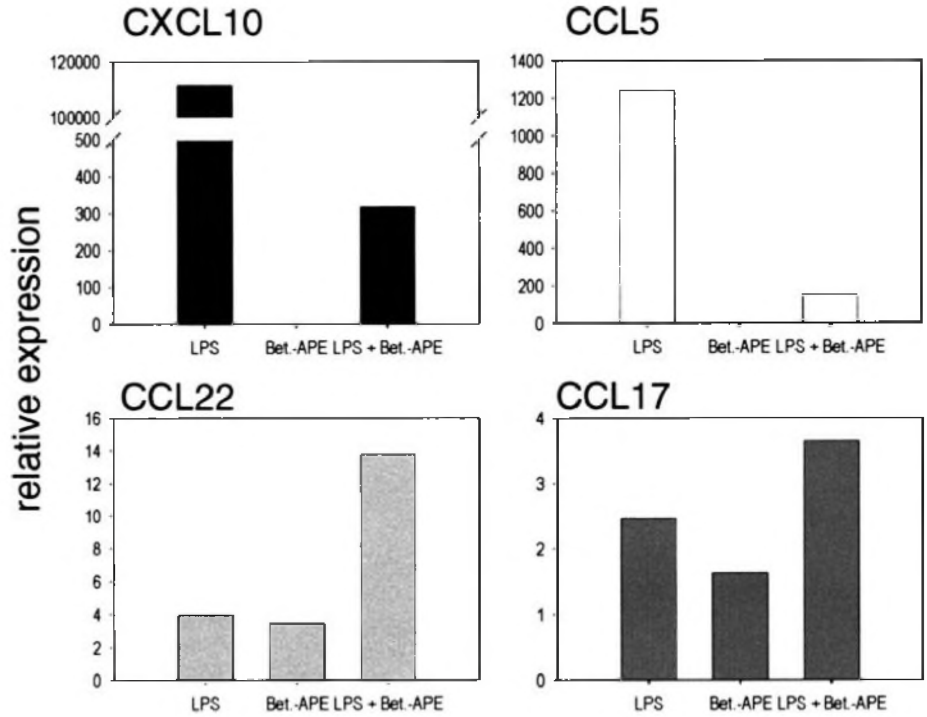

FIGURE 3. *Bet.*-APE block the LPS-induced CCL5 and CXCL10 m-RNA expression, whereas LPS-induced CCL22 and CCL17 expressions are enhanced. Human MoDCs were stimulated for 6 h with LPS (100 ng/ml) and/or *Bet.*-APE (3 mg/ml), processed for RNA extraction, and analyzed by real-time PCR for the expression of CXCL10 (IP-10), CCL5 (RANTES), CCL22 (MDC), and CCL17 (TARC). Data are expressed as relative expression ($2^{(-\Delta\Delta CT)}$) and one representative experiment of five performed is shown.

migration assay, 100 μl of the cell suspension were pipetted into the upper and the diluted chemokines (CXCL12, CCL19, medium as control) into the lower chamber of the Transwell.

After 1 h of incubation at 37°C with 5% carbon dioxide, cells that transmigrated into the lower chamber were recovered and acquired with a FACSCalibur device for 60 s at a flow rate of 60 μl/min. Data acquisition and analysis were restricted to events with the forward and side scatter properties of cells and not cell debris.

Chemokine production of DC

MoDCs were harvested, seeded into 96-well plates (Nunc) at a density of 100,000 cells/well, and treated with medium or 100 ng/ml LPS, alone or in combination with different concentrations of *Bet.*-APE. After 24 h, supernatants were analyzed for the presence of CXCL10/IP-10, CCL5/RANTES, CCL22/MDC and CCL17/TARC by ELISA. ELISA kits used were from BD Biosciences (CXCL10) and R&D Pharmaceuticals (CCL5, CCL22, CCL17).

cAMP measurements

DC, generated as decribed above, were stimulated at 10^5 cells/100 μl/well in 96-well flat-bottom culture plates (Nunc) with various concentrations of PGE_2 (Cayman) or *Bet.*-APE. After 40 min, cells were lysed by addition of equal volumes of sample/lysis buffer (cAMP-Screen System; Applied Biosystems), resuspension, and incubation for 30 min at 37°C. The lysates were then analyzed for cAMP by ELISA (cAMP-Screen System) following the supplier's instructions.

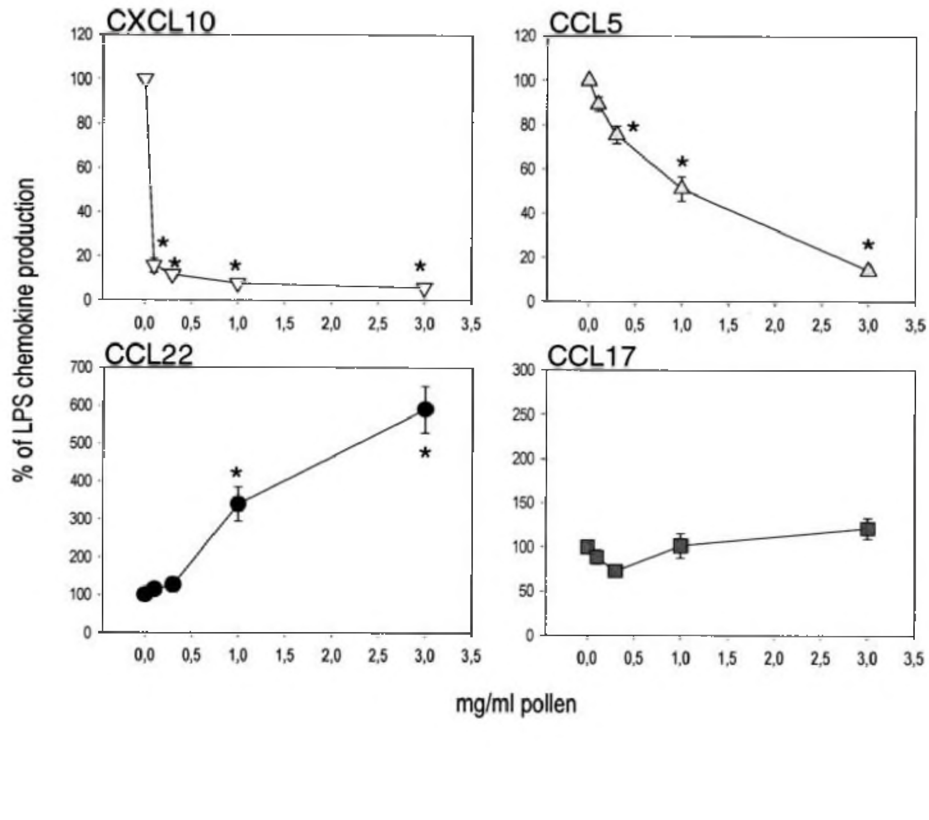

FIGURE 4. *Bet.*-APE inhibit the LPS-induced CXCL10 and CCL5 release and augment LPS-induced CCL22 secretion in a dose-dependent manner, whereas CCL17 production is not affected. Human MoDCs were stimulated with LPS (100 ng/ml) and graded concentrations of *Bet.*-APE (0.1 to 3 mg/ml). After 24 h, cell-free supernatants were harvested and analyzed by ELISA for CXCL10 (IP-10), CCL5 (RANTES), CCL22 (MDC) and CCL17 (TARC). Differences in LPS-stimulated DCs in the presence of *Bet.*-APE were significant ($p < 0.05$) for CXCL10 at a concentration of 0.1 mg/ml, for CCL5 at 0.3 mg/ml, and for CCL22 at 1 mg/ml, whereas LPS-induced CCL17 release did not change significantly in the presence of *Bet.*-APE. Results are given as the percentage (mean ± SD) of LPS-induced chemokine production (CXCL10 x = 17,766.7 ± 2,786.3 pg/ml; CCL5 x = 12,766.7 ± 2,254.6; CCL22 x = 4,666.7 ± 723.6; CCL17 x = 15,136 ± 4,265.3). *, Significant changes ($p \leq 0.05$). Values are for one representative experiment of three performed in triplicate.

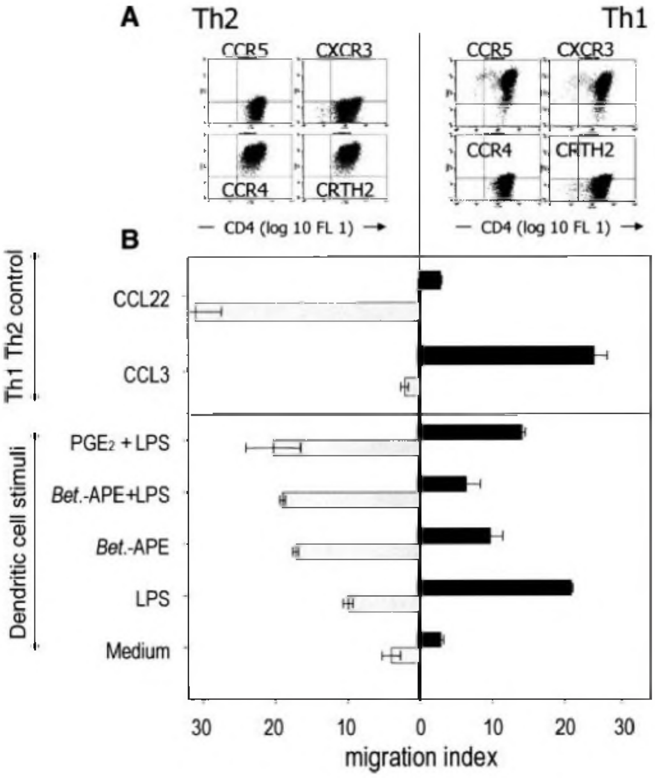

FIGURE 5. *Bet.*-APE inhibits the capacity of human MoDCs to attract Th1 and augments their Th2-attracting activity. *A*, Polarized T cell clones were obtained from T cell lines from mixed lymphocyte reactions. To evaluate successful polarization, T cell clones were stained for CCR4, CRTh2 (marker for Th2 cells) and CXCR3 and CCR5 (marker for Th1 cells). Shown is the flow cytometry of two representative clones double-stained with FITC-conjugated mAbs to CD4 (*x*-axis) and the PE-conjugated chemokine receptors (*y*-axis). *B*, DCs were stimulated for 24 h with LPS together with *Bet.*-APE or *Bet.*-APE alone and LPS plus PGE_2 as positive control. Cell-free supernatants diluted 1/100 in complete medium and 0.5% BSA were used for cell migration studies of polarized Th1 and Th2 T cell clones. Polarized T cell clones (1×10^5) were incubated in the upper chamber in 24-well Transwell chambers; indicated DC-supernatants were given in the lower chamber. In each experiment, positive controls for a Th1 (CCL3, 100 ng/ml) and a Th2 (CCL22, 100 ng/ml) chemokine were added. Data are given as migration index ± SD (migration index: ratio of cells migrated to chemoattractant or DC-supernatant and medium). Values are for one representative experiment of three performed in triplicate.

Preparation of E_1-phytoprostanes

Racemic E_1-phytoprostanes were prepared by autooxidation of α-linolenic acid and purified as described previously (15).

Statistics

Student's paired *t* test was used to compare differences in chemokine release, cAMP levels and cell migration. *p* values of 0.05 or less were considered to indicate significance.

Results

Bet.*-APE induces CXCR4 and down-regulates CCR1 and CCR5 expression*

Maturation of DCs results in substantial changes in the expression of chemokine receptors and the ability to migrate toward chemokine gradients. Therefore, expression of selected chemokine receptors and its modulation by *Bet.*-APE on human MoDCs was analyzed at mRNA (real time PCR) and protein level (flow cytometry). LPS-induced DC maturation, as determined by CD83 expression, was accompanied by a down-regulation of CCR1 and CCR5 and an up-

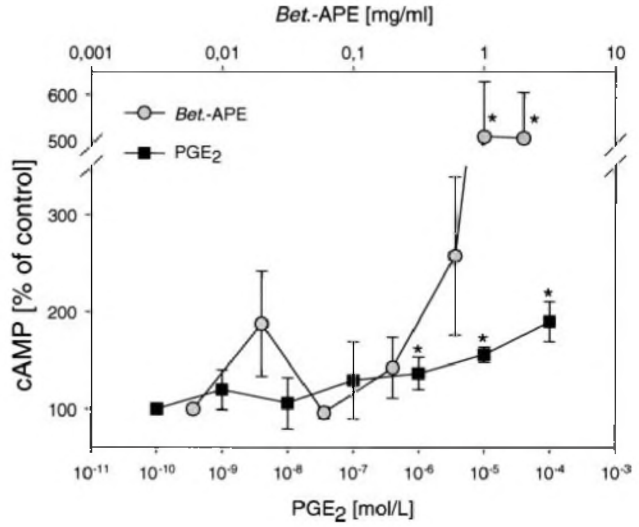

FIGURE 6. *Bet.*-APE induces intracellular cAMP levels in MoDCs. Human MoDCs were harvested on day 5 of culture and treated for 40 min with medium or the indicated concentrations of PGE_2 or *Bet.*-APE, respectively. Cell lysates were analyzed for cAMP by ELISA. Data are shown as percent of cAMP levels of medium-treated cells. Values are the mean ± SD of five independent experiments, each performed in triplicates.

regulation of CXCR4 (Fig. 1*A*). Even though stimulation with *Bet.*-APE did not induce a significant DC maturation (CD83), it still led to a down-regulation of CCR1 and CCR5 and a pronounced up-regulation of CXCR4 surface expression on immature DCs. Chemokine receptor mRNA expression was analyzed by real-time PCR (Fig. 1*B*). LPS treatment resulted in a moderate down-regulation of CCR1 and CCR5 mRNA expression whereas CXCR4 mRNA was 3-fold up-regulated. Consistent with flow cytometric analysis of receptor surface expression, stimulation with *Bet.*-APE alone led to reduced levels of CCR1 and CCR5 mRNA and a substantial induction of CXCR4 mRNA. In addition, *Bet.*-APE had an additive effect on the CXCR4 mRNA up-regulation induced by LPS.

Bet.*-APEs enhance migration of DCs toward the CXCR4 ligand, CXCL12*

The functional activity of chemokine receptors was examined by measuring the migration to ligands of CCR1, CCR5, CCR7, and CXCR4. DCs were stimulated with LPS and/or *Bet.*-APE (3 mg/ml). Because the presence of PGE_2 has been described to be important for the response to the CCR7 and CXCR4 ligands (17, 18), DCs were stimulated with PGE_2 (1×10^{-5}) plus LPS or PGE_2 alone as positive control. Immature DCs showed a baseline, non-directional migration to all stimuli used that was comparable with that of medium control. After LPS stimulation, baseline migration to medium and migration toward the CCR1 and CCR5 ligands (CCL16 and CCL4, respectively) was absent, whereas high migratory activity was observed to the CCR7 ligands CCL21 and CCL19. The CXCR4 ligand CXCL12 induced only a weak migratory response in LPS-stimulated DCs. When PGE_2 was added to the LPS-stimulated culture, the migration toward CCL19 and CCL21 was enhanced 2.2 and 1.2-fold, respectively. Notably, PGE_2 enhanced the migration of LPS-stimulated DCs toward CXCL12 55-fold. Similarly, when DCs were costimulated with LPS and *Bet.*-APE, the migration toward CXCR4 ligand CXCL12 was enhanced 37-fold. In contrast, the migration to the CCR7 ligands CCL21 and CCL19, although enhanced, did not change significantly when *Bet.*-APE was added. The stimulation with PGE_2 or *Bet.*-APE in the absence of LPS led only to a marginal variation

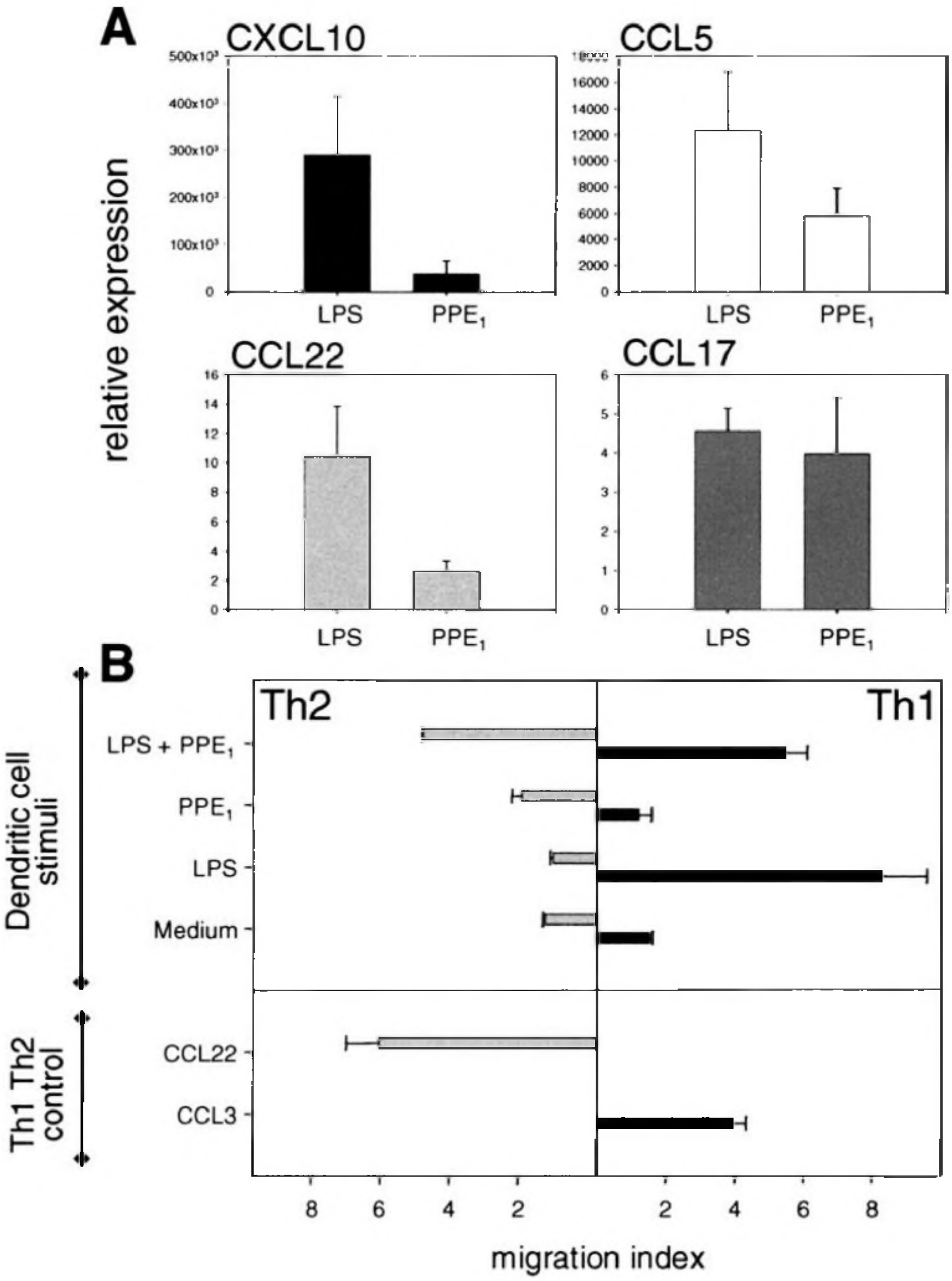

FIGURE 7. PPE_1 blocks LPS-induced CCL5, CXCL10, and CCL22 expression and leads to reduced Th1 and enhanced Th2 attraction via DCs. *A*, Human MoDCs were stimulated for 6 h with E_1-phyoprostanes (1×10^{-5} M) in the presence of LPS (100 ng/ml). Analysis of chemokine expression was performed by real-time PCR. Data are expressed as relative expression ($2^{(-\Delta\Delta CT)}$) of three independent experiments performed in triplicate. *B*, DCs were stimulated for 24 h with LPS alone or together with PPE_1 1×10^{-5} M. Cell-free supernatants were used for cell migration studies of polarized Th1 and Th2 T cell clones. In each experiment, positive controls for a Th1 (CCL3, 100 ng/ml) and a Th2 (CCL22, 100 ng/ml) chemokine were added. Data are given as migration index ± SD. Migration index: ratio of cells migrated to chemoattractant or DC-supernatant and medium. Values are for one representative experiment of three performed in triplicate.

in the random migration but not in the directed migration toward on of the tested ligands.

The enhanced migration of Bet.*-APE-stimulated DCs toward CXCL12 is dependent on the induction of adenylyl cyclase*

We observed an enhanced chemotaxis of DC toward the Th2 chemokine CXCL12 after simultaneous stimulation with LPS and either PGE_2 or *Bet.*-APE (Fig. 2*B*). Because PGE_2 is a known inducer of adenylyl cyclase, we investigated whether the enhanced tendency of DC to migrate toward CXCL12 when stimulated with LPS and *Bet.*-APE might be due to elevated levels of the second messenger cyclic AMP (cAMP). DC migration to chemokines was assessed in the absence or presence of an inhibitor of adenylyl cyclase, SQ22536. As a positive control for cAMP induction, forskolin, an adenylyl cyclase inducer, was included as stimulus. LPS treatment alone led to migration toward CCL19, whereas no chemotaxis occurred toward CXCL12 (Fig. 2, *B* and *C*). Interestingly, the cAMP agonist forskolin significantly induced the migration of LPS-stimulated DC toward CXCL12 and enhanced the migrations toward the CCR7 ligand CCL19, as did *Bet.*-APE (Fig. 2, *B* and *C*). This effect of forskolin or *Bet.*-APE on the migratory behavior of LPS-stimulated DCs was significantly reversed in the presence of SQ-22536. The CCL19-responsivness was even more sensitive to the cAMP antagonist than CXCL12. Comparable results were obtained with the combined stimulation of DCs with LPS and PGE_2 (data not shown). Yet unexplained is the induction of the CXCL19 responsiveness of LPS-stimulated DCs in the presence of the cAMP-antagonist SQ22536.

Bet.*-APEs up-regulate DC production of Th2-chemokines, whereas Th1 chemokines are blocked*

In the next series of experiments, we investigated the effect of *Bet.*-APE on chemokine production by DCs. Consistent with previous observations (19, 20), immature DCs constitutively produced CCL22 and CCL17 (532.6 ± 96.3 pg/ml and 682.6 ± 78.4

pg/ml, respectively) but not CXCL10 or CCL5, as determined by quantitative RT-PCR and ELISA. Treatment of immature DC with *Bet.*-APE significantly increased the mRNA expression of CCL22 and CCL17, whereas CXCL10 or CCL5 were not induced. Moreover, *Bet.*-APE increased the LPS-induced expression of CCL22 and CCL17 and strongly inhibited CXCL10 and CCL5 induced by LPS (Fig. 3). The mRNA data were confirmed at protein level analyzing 48-h supernatants of DCs. Using graded concentrations of *Bet.*-APE (0.1–3 mg/ml), the suppression of LPS-induced CXCL10 release was already significant at 0.1 mg/ml whereas CCL5 reduction became significant at 0.3 mg/ml (Fig. 4). The enhancement of CCL22 was evident at a *Bet.*-APE dose of 1 mg/ml whereas LPS-induced CCL17 protein release did not change significantly in the presence of *Bet.*-APE (Fig. 4). Comparable results were achieved with CD40L stimulation (data not shown).

Bet.*-APEs enhance the capacity of DCs to attract type 2 T lymphocytes*

Depending on their polarization, T lymphocytes differentially express receptors for CXCL10, CCL5, CCL22, and CCL17, with type 1 cells preferentially expressing CXCR3 and CCR5 and type 2 cells expressing CCR3, CCR4, and CRTh2 (6, 21–23). We thus investigated whether *Bet.*-APE could affect the capacity of DCs to attract type 1 and type 2 T lymphocytes. Polarized T cell clones were generated and characterized for IL-4 and IFN-γ production and for expression of CXCR3, CCR5, CCR3, and CRTh2. As expected, type 1 lymphocytes showed high levels of IFN-γ and low levels of IL-4, whereas type 2 cells had high IL-4 and low IFN-γ production (data not shown). CCR5 and CXCR3 were expressed by Th1 clones, whereas CCR4 and CRTh2 were present on type 2 T cell clones (Fig. 5*A*). Furthermore, Th2 clones showed a significant migration (migration index, >2) to CCL22, whereas migration to CCL3 was negative. Equally, Th1 clones migrated significantly to CCL3 but not to CCL22 (Fig. 5*B*). As expected, both Th1 and Th2 T cell clones showed a higher migratory response to supernatants from LPS-stimulated DCs than did those from nonstimulated immature DCs. Here, Th1 cells migrated more efficiently than Th2 cells to supernatants from LPS-stimulated DCs (Fig. 5*B*). In contrast, supernatants from *Bet.*-APE-treated DCs induced a preferential induction of Th2 cells. This was consistent with the finding that *Bet.*-APE alone was sufficient to induce DC production of CCL22. In line with the inhibitory effect of *Bet.*-APE on the LPS-induced production of CXCL10 and CCL5, *Bet.*-APE stimulation reduced the capacity of LPS-stimulated DCs to attract Th1 cells. The immunomodulatory capacity of *Bet.*-APE on DC chemokine production and migratory response of Th1 and Th2 cells was comparable with that of PGE_2, well known to induce in DCs the release of Th2 chemokines (24).

Bet.*-APEs enhance cAMP level in human MoDCs*

PGE_2 exerts its immunomodulatory effects on DC chemokine expression via its receptors, EP2 and EP4 (25), both of which signal primarily via the cAMP/protein kinase A pathway. To investigate whether an induction of the intracellular cAMP level might also be involved in the observed modulation of chemokine expression by aqueous pollen extracts, cAMP was measured in lysates of DCs stimulated with various concentrations of PGE_2 or *Bet.*-APE. Although PGE_2 treatment led to only a 2-fold induction of intracellular cAMP, *Bet.*-APE dose-dependently increased cAMP levels up to 7-fold (Fig. 6).

E_1-Phytoprostanes block LPS-induced Th1 chemokines in DCs and consequently lead to reduced Th1 attraction and enhanced Th2 attraction compared with LPS-stimulated DCs

We recently demonstrated the presence of phytoprostanes with a predominance of PPE_1 in aqueous birch pollen extracts (*Bet.*-APE). E_1-phytoprostanes, similar to *Bet.*-APE, dose-dependently inhibited IL-12 production and induced an increased Th2-polarizing capacity of human MoDCs (15). To evaluate whether PPE_1 was also responsible for the reduction of Th1 and induction of Th2 chemokines, DCs were stimulated with PPE_1 (3×10^{-8}–1×10^{-5} M) together with LPS. Here, PPE_1 lead to a significant reduction of LPS-induced CXCL10, CCL5, and CCL22 at the concentration of 1×10^{-5} M (Fig. 7*A*). LPS-induced CCL17 did not change significantly in the presence of PPE_1, being neither enhanced nor blocked. On a functional level, DCs stimulated with PPE_1 alone (1×10^{-5} M) showed a similar attraction pattern of Th1 and Th2 as nonstimulated DCs. However, LPS plus PPE_1 (1×10^{-5} M) stimulated DCs emerged as low Th1 and strong Th2 attracting cells compared with LPS-stimulated DCs (Fig. 7*B*). This points to the fact that PPE_1 is one player in the Th2-polarizing effect of *Bet.*-APE albeit not being the only one. The CCL22 inducing factor remains elusive to date.

Discussion

In the current study, water-soluble factors from pollen were found to be a critical switch factor for the acquisition of migratory capacity and T cell attraction profile of DCs. This underlines a novel role of pollen in the decision-making process of allergic responses that goes beyond the allergen carrier model. *Bet.*-APE lead, together with LPS, to an up-regulation of the lymphoid chemokine receptor CXCR4 and the coordinated down-regulation of CCR1 and CCR5. Functionally, DCs exposed to *Bet.*-APE plus LPS migrated vigorously to CCL19 and CCL21, whereas chemotaxis to CCL16 and CCL4 was reduced compared with immature DCs. *Bet.*-APE alone induced CXCR4 expression at mRNA and protein level which, however, appeared to be nonfunctional because no significant migration toward CXCL12 was observed. However, *Bet.*-APE stimulation in the presence of a maturational signal such as LPS induced in DCs a strong migratory response to the CXCR4 ligand CXCL12. Because CXCL12 is constitutively expressed in lymphoid tissue (26), this could be critical for maintenance of DC levels in such tissues during allergic inflammation. In line with previous studies (6, 27), DC stimulation with LPS alone also induced a moderate up-regulation of CXCR4, which did, however, not lead to a significant migration to CXCL12. PGE_2 has recently been described to be required for human DC migration turning CXCR4 into a functionally expressed receptor (7). Also in our hands, LPS-stimulated DCs migrated only after costimulaton with PGE_2 toward the CXCR4 ligand CXCL12. Also, pollen extracts contain a factor turning the CXCR4 expression of DCs in to a functional receptor, which in turn can favor DC migration to lymphoid organs (28–30). It has previously been reported that cholera toxin induces DC maturation that is associated with membrane CXCR4 expression (27). Cholera toxin may lead to CXCR4 expression on DC membranes by elevating the intracellular levels of cAMP. Consistent with this hypothesis, Cole et al. (31) reported that cAMP up-regulates membrane CXCR4 expression on lymphocytes by decreasing receptor internalization without affecting the level of gene expression. The observed chemotaxis toward CXCL12 of DCs stimulated with LPS and PGE_2, forskolin or *Bet.*-APE was reduced significantly in the presence of an adenylyl cyclase inhibitor. Consistent with this finding, *Bet.*-APE led to a rapid and significant up-regulation of cAMP in DCs. This suggests that water-soluble factors from pollen bind to a cAMP-coupled receptor that may account for the observed chemokine receptor regulation. Whether the recently described prostaglandin-like, pollen-associated phytoprostanes (15, 32, 33) are responsible for this effects is currently under investigation.

Chemokines regulate leukocyte trafficking by inducing firm integrin-dependent adhesion of blood leukocytes to endothelial cells and by inducing directional migration. Because pollen modifies the capacity of mature DCs to produce IL-12 and consequently the outcome of the ensuing Th cell polarization (15), we wanted to assess whether they also might induce effects on the ability of DCs to attract different polarized Th cells. Indeed, *Bet.*-APE affected the pattern of chemokine release from DCs by up-regulating the constitutive production of CCL22 and CCL17 whereas Th1 chemokines such as CXCL10 and CCL5 were not induced by *Bet.*-APE alone. Furthermore, *Bet.*-APE substantially inhibited the LPS-induced secretion of CXCL10 and CCL5, whereas CCL22 release was up to 6-fold increased. The pattern of chemokine release from DCs matured in the presence of *Bet.*-APE suggested an altered capacity of DCs to attract T cell subsets, because *Bet.*-APE up-regulate the production of the Th2-related chemokine CCL22 and inhibit the release of the LPS-induced Th1 chemokines CXCL10 and CCL5. To assess whether *Bet.*-APE might enhance Th2 recruitment, we tested the capacity of supernatants from DC cultures to induce migration of type 1 or type 2 polarized T cells.

We found that DCs matured in the presence of *Bet.*-APE attracted type 1 polarized Th1 clones less efficiently then Th2 clones. Furthermore, *Bet.*-APE blocked the LPS-induced chemoattraction of Th1 cells, and increased the LPS-induced attraction of Th2 cells. These results confirm the hypothesis that DCs exposed to *Bet.*-APE have a diminished capacity to amplify type 1 immune responses (15) and at the same time favor the attraction of type 2 Th cells to the site of allergic inflammation.

Several data indicate that PGE_2 up-regulates CCL22 production by LPS or CD40L-stimulated DCs (24, 34). Furthermore, reports indicate that PGE_2 also suppresses chemokine mRNA expression and chemokine production in various types of cells. For example, PGE_2 or intracellular accumulation of cAMP suppresses CCL5 production by murine mesangial cells (35) and CXCL10 mRNA expression by cultured keratinocytes (36). In our hands, the stimulation of DCs with LPS and PGE_2 lead to a preferential attraction of Th2 cells, confirming previous data showing that PGE_2 enhances LPS-induced Th2 chemokines (31). As already mentioned, PGE_2 exerts its immunomodulatory effects on DC chemokine expression via its receptors, EP2 and EP4 (25), both of which are coupled to the induction of adenylyl cyclase and a consecutive rise in intracellular cAMP. Interestingly, the increase in intracellular cAMP levels by *Bet.*-APE in DCs was even more pronounced than that induced by PGE_2. The observed up-regulation of CCL22 by stimulation with LPS and *Bet.*-APE together with the regulation of CXCR4 indicates that *Bet.*-APE contains bioactive compounds with immunomodulatory characteristics similar to that of PGE_2, most probably acting via a cAMP-dependent mechanism.

To find the responsible substance for the observed effects, E_1-phytoprostanes were tested in our DC system. Here we demonstrate that PPE_1 accounts in part for the observed effects such as the reduction of CXCL10 and CCL5. However, it is not responsible for the CCL22 induction, pointing to the fact that the in vitro effects of *Bet.*-APE very likely reflect summative effects of various substances, which may act synergistically in the Th2-polarizing capacity of *Bet.*-APE. Aqueous pollen extracts contain a large number of different substances with potential immunomodulatory capacities. Besides pollen-associated lipid mediators such as phytoprostanes and oxilipins, carbohydrates or proteins might also exert Th-polarizing effects.

In conclusion, we have demonstrated that water-soluble mediators from pollen change the chemokine receptor profile of DCs, which may result in an enhanced capacity to localize to lymph nodes. In addition, we have described a mechanism by which pollen can promote immune deviation toward a type 2 response, i.e., by preventing DC recruitment of type 1 and enhancing the attraction of type 2 T lymphocytes. These findings support the hypothesis that pollen grains themselves appear to harbor important tools to pave the way toward a Th2-dominated immune response against pollen-associated allergens.

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Disclosures

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References

1. Jakob, T., C. Traidl-Hoffmann, and H. Behrendt. 2002. Dendritic cells: the link between innate and adaptive immunity in allergy. *Curr. Allergy Asthma Rep.* 2: 93–95.
2. Banchereau, J., and R. M. Steinman. 1998. Dendritic cells and the control of immunity. *Nature* 392: 245–252.
3. Sallusto, F., and A. Lanzavecchia. 2000. Understanding dendritic cell and T-lymphocyte traffic through the analysis of chemokine receptor expression. *Immunol. Rev.* 177: 134–140.
4. Sallusto, F., B. Palermo, D. Lenig, M. Miettinen, S. Matikainen, I. Julkunene, R. Forster, R. Burgstahler, M. Lipp, and A. Lanzavecchia. 1999. Distinct patterns and kinetics of chemokine production regulate dendritic cell function. *Eur. J. Immunol.* 29: 1617–1625.
5. Dieu, M. C., B. Vanbervliet, A. Vicari, J. M. Bridon, E. Oldham, S. Ait-Yahia, F. Briere, A. Zlotnik, S. Lebecque, and C. Caux. 1998. Selective recruitment of immature and mature dendritic cells by distinct chemokines expressed in different anatomic sites. *J. Exp. Med.* 188: 373–386.
6. Sallusto, F., P. Shaerli, P. Loetscher, C. Schaniel, D. Lenig, C. R. Mackay, S. Oin, and A. Lanzavecchia. 1998. Rapid and coordinated switch in chemokine receptor expression during dendritic cell maturation. *Eur. J. Immunol.* 28: 2760–2769.
7. Legler, D. F., P. Krause, E. Scandella, E. Singer, and M.Groettrup. 2006. Prostaglandin E_2 is generally required for human dendritic cell migration and exerts its effect via EP2 and EP4 receptors. *J. Immunol.* 176: 966–973.
8. Wierenga, E. A., M. Snoek, C. de Groot, I. Chretien, J. D. Bos, H. M. Jansen, and M. L. Kapsenberg. 1990. Evidence for compartmentalization of functional subsets of $CD4^+$ T lymphocytes in atopic patients. *J. Immunol.* 144: 4651–4656.
9. Schappi, G. F., C. Suphioglu, P. E. Taylor, and R. B Knox. 1997. Concentrations of the major birch tree allergen Bet v 1 in pollen and respirable fine particles in the atmosphere. *J. Allergy Clin. Immunol.* 100: 656–661.
10. Traidl-Hoffmann, C., A. Kasche, A. Menzel, T. Jakob, M. Thiel, J. Ring, and H. Behrendt. 2003. Impact of pollen on human health: more than allergen carriers? *Int. Arch. Allergy Immunol.* 131: 1–13.
11. Behrendt, H., and W. M. Becker. 2001. Localization, release and bioavailability of pollen allergens: the influence of environmental factors. *Curr. Opin. Immunol.* 13: 709–715.
12. Behrendt, H., A. Kasche, C. Ebner von Eschenbach, U. Risse, J. Huss-Marp, and J. Ring. 2001. Secretion of proinflammatory eicosanoid-like substances precedes allergen release from pollen grains in the initiation of allergic sensitization. *Int. Arch. Allergy Immunol.* 124: 121–125.
13. Traidl-Hoffmann, C., A. Kasche, T. Jakob, M. Huger, S. Plötz, I. Feussner, J. Ring, and H. Behrendt. 2002. Lipid mediators from pollen act as chemoattractants and activators of polymorphonuclear granulocytes. *J. Allergy Clin. Immunol.* 109: 831–838.
14. Plötz, S., C. Traidl-Hoffmann, I. Feussner, A. Kasche, A. Feser, J. Ring, T. Jakob, and H. Behrendt. 2004. Chemotaxis and activation of human peripheral blood eosinophils induced by pollen associated lipid mediators. *J. Allergy Clin. Immunol.* 2113: 1152–1160.
15. Traidl-Hoffmann, C., V. Mariani, H. Hochrein, K. Kark, H. Wagner, J. Ring, M. J. Mueller, T. Jakob, and H. Behrendt. 2005. Pollen-associated phytoprostanes inhibit dendritic cell interleukin-12 production and augment T helper type 2 cell polarization. *J. Exp. Med.* 201: 627–636.
16. Sallusto, F., and A. Lanzavecchia. 1994. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor α. *J. Exp. Med.* 179: 1109–1118.
17. Luft, T., M. Jefford, P. Luetjens, T. Toy, H. Hochrein, K. A. Masterman, C. Maliszewski, K. Shortman, J. Cebon, and E. Maraskovsky. 2002. Functionally distinct dendritic cell (DC) populations induced by physiologic stimuli: prostaglandin E_2 regulates the migratory capacity of specific DC subsets. *Blood* 100: 1362–1372.

18. Scandella, E., Y. Men, D. F. Legler, S. Gillessen, L. Prikler, B. Ludewig, and M. Groettrup. 2004. CCL19/CCL21-triggered signal transduction and migration of dendritic cells requires prostaglandin E2. *Blood* 103: 1595–1601.
19. Corinti, S., D. Medaglin, A. Cavani, M. Rescigno, G. Pozzi, P. Ricciardi-Castagnoli, and G. Girolomoni. 1999. Human dendritic cells very efficiently present a heterologous protein antigen expressed on the surface of recombinant Gram-positive bacteria to $CD4^+$ T lymphocytes. *J. Immunol.* 163: 3029–3036.
20. Vulcano, M., C. Albanesi, A. Stoppacciaro, R. Bagnati, G. D'Amico, S. Struyf, P. Transidico, R. Bonecchi, A. Del Prete, P. Allavena, et al. 2001. Dendritic cells as a major source of macrophage-derived chemokine/CCL22 in vitro and in vivo. *Eur. J. Immunol.* 31: 812–822.
21. Sallusto, F., D. Lenig, C. R. Mackay, and A. Lanzavecchia. 1998. Flexible programs of chemokine receptor expression on human polarized T helper 1 and 2 lymphocytes. *J. Exp. Med.* 187: 875–883.
22. Bonecchi, R., G. Bianchi, P. P. Bordignon, D. D'Ambrosio, R. Lang, A. Borsatti, S. Sozzani, P. Allavena, P. A. Gray, A. Mantovani, and F. Sinigaglia. 1998. Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. *J. Exp. Med.* 187: 129–134.
23. Zingoni, A., H. Soto, J. A. Hedrick, A. Stoppacciaro, C. T. Storlazzi, F. Sinigaglia, D. D'Ambrosio, A. O'Garra, D. Robinson, M. Rocchi, et al. 1998. The chemokine receptor CCR8 is preferentially expressed in Th2 but not in Th1 cells. *J. Immunol.* 161: 547–551.
24. McIlroy, A., G. Caron, S. Blanchard, I. Fremaux, D. Duluc, Y. Delneste, A. Chevailler, and P. Jeannin. 2006. Histamine and prostaglandin E up-regulate the production of Th2-attracting chemokines (CCL17 and CCL22) and down-regulate IFN-γ-induced CXCL10 production by immature human dendritic cells. *Immunology* 117: 507–516.
25. Jing, H., J. H. Yen, and D. Ganea. 2004. A novel signaling pathway mediates the inhibition of CCL3/4 expression by prostaglandin E_2. *J. Biol. Chem.* 279: 55176–55186.
26. Bleul, C. C., R. C. Fuhlbrigge, J. M. Casasnovas, A. Aiuti, and T. A. Springer. 1996. A highly efficacious lymphocyte chemoattractant, stromal cell-derived factor 1 (SDF-1). *J. Exp. Med.* 184: 1101–1109.
27. Gagliardi, M. C., F. Sallusto, M. Marinaro, A. Langenkamp, A. Lanzavecchia, and M. T. De Magistris. 2000. Cholera toxin induces maturation of human dendritic cells and licences them for Th2 priming. *Eur. J. Immunol.* 30: 2394–2403.
28. Lanzavecchia, A., and F. Sallusto. 2001. The instructive role of dendritic cells on T cell responses: lineages, plasticity and kinetics. *Curr. Opin. Immunol.* 13: 291–298.
29. Sozzani, S., P. Allavena, G. D'Amico, W. Luini, G. Bianchi, M. Kataura, T. Imai, O. Yoshie, R. Bonecchi, and A. Mantovani. 1998. Differential regulation of chemokine receptors during dendritic cell maturation: a model for their trafficking properties. *J. Immunol.* 161: 1083–1086.
30. Sallusto, F., C. R. Mackay, and A. Lanzavecchia. 2000. The role of chemokine receptors in primary, effector, and memory immune responses. *Annu. Rev. Immunol.* 18: 593–620.
31. Cole, S. W., B. D. Jamieson, and J. A. Jack. 1999. CAMP up-regulates cell surface expression of lymphocyte CXCR4: implications for chemotaxis and HIV-1 infection. *J. Immunol.* 162: 1392–1400.
32. Mueller, M. J. 1998. Radically novel prostaglandins in animals and plants: the isoprostanes. *Chem Biol.* 5: 323–333.
33. Parchmann, S., and M. J. Mueller. 1998. Evidence for the formation of dinor isoprostanes E1 from α-linolenic acid in plants. *J. Biol. Chem.* 273: 32650–32655.
34. Kuroda, E., T. Suqiura, K. Okada, K. Zeki, and U. Yamashita. 2001. Prostaglandin E_2 up-regulates macrophage-derived chemokine production but suppresses IFN-inducible protein-10 production by APC. *J. Immunol.* 166: 1650–1658.
35. Satriano, J. A., B. Banas, P. Nelson, and D. O. Schlöndorff. 1997. Regulation of RANTES and ICAM-1 expression in murine mesangial cells. *J. Am. Soc. Nephrol.* 8: 596–599.
36. Boorsma, D. M., J. Flier, E. N. van den Brink, S. Sampat, H. L. Walg, R. Willemze, C.P . Tensen, and T. J. Stoof. 1999. IP-10 mRNA expression in cultured keratinocytes is suppressed by inhibition of protein kinase C and tyrosine kinase and elevation of cAMP. *Cytokine* 11: 469–502.